Faculty of Resource Science and Technology

MOLECULAR CLONING AND CHARACTERIZATION OF PARTIAL cDNA ENCODING FOR ALPHA-AMYLASE INHIBITOR FROM KELAMPAYAN (*Neolamarckia cadamba*)

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DECLARATION

I hereby declare that this thesis is based on original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

A	Ampere
α-amylase	Alpha-amylase
AGE	Agarose gel-electrophoresis
bp	Base pair(s)
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
ddH_2O	Double-distilled water
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
EST	Expressed Sequence Tags
mRNA	Messenger RNA
min	Minute
NCBI	National Centre for Biotechnology Information
µl	Microliter
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
Rpm	Revolution per minute

RT	Reverse Transcription
sec	Seconds
Ta	Annealing temperature
Tm	Melting temperature
TAE	Tris-Acetate EDTA
V	Volt

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ABSTRACT

Pest and pathogen infections are the main problems faced by forest plantations. Plants have developed pest resistance during evolution to enable it to survive. One of the natural protective mechanisms is known as alpha-amylase inhibitors. Alpha-amylase inhibitors function to inhibit the activities of alpha-amylase located in the insect's gut. *Neolamarckia cadamba* or also known as Kelampayan is selected as the tree species due to its fast growth rate and wide range of usage and values. Besides that, no research has been done to identify the presence of alpha-amylase inhibitor gene in *N. cadamba*. The objective of this study was to clone and characterize the partial cDNA encoding alpha-amylase inhibitor from *N. cadamba*. Total RNA was isolated from developing xylem tissues and leaves. Total RNA was reversed transcribed into cDNA through RT-PCR. The purified PCR product was then sent for sequencing. Good sequencing data was not obtained and thus BLASTn cannot be performed to identify the identity of the gene.

Keywords: *Neolamarckia cadamba* (Kelampayan), alpha-amylase inhibitor, cDNA, RT-PCR, cloning

ABSTRAK

Serangga perosak merupakan masalah utama yang dihadapi oleh pertumbuhan hutan. Tumbuhan mempunyai sistem pertahanan selepas evolusi. Salah satu sistem pertahanan secara semula jadi adalah dinamakan sebagai alfa-amilase inhibitors. Alfa-amilase inhibitors blok dan menghalang pencernaan alfa-amilase yang didapati dalam usus serangga. Neolamarckia cadamba atau dinamakan sebagai Kelampayan digunakan dalam kajian ini atas sebab kadar pertumbuhan yang pantas dan mempunyai kepentingan dan kegunaan yang pelbagai. Selain itu, tiada kajian yang dibuat atas gen alfa-amilase inhibitors ini. Objektif kajian ini ialah mengklon dan mengcirikan cDNA separa yang menyandi gen alfa-amilase inhibitors pada pokok N. cadamba. RNA keseluruhan diasingkan dari tisu xylem dan daun. RNA keseluruhan selepasnya telah ditranskripsi ke cDNA melalui RT-PCR. Produk PCR kemudian dihantar untuk sequencing. Data sequencing yang baik tidak berjaya didapati dan menyebabkan BLASTn tidak boleh dijalankan untuk mengesan gen identity.

Kata kunci: *Neolamarckia cadamba (Kelampayan), alfa-amilase inhibitor, cDNA, RT-PCR, klonin*

1.0 INTRODUCTION

Recently, rapid increase of global population has lead to high demand of forest products such as fuel, fibre, timber and other industries (Bradshaw *et al.*, 1989). According to PERKASA (2012), *Acacia mangium* (Mangium) , *Acacia hybrid* (Acacia), *Hevea brasiliensis* (rubber), *N. cadamba* (Kelampayan), *Azadirachta excelsea* (Sentang), *Eucalyptus pellita* (Eucalyptus), *E. deglupta* (Eucalyptus), *E. grandis* (Eucalyptus), *Paraserianthes falcataria* (Batai) and *Shores macophylia* (Engkabang Jantung) are the examples of forest plantation species selected by the government. The study of molecular biology of forest trees enables a better understanding of the many natural populations of woody trees (Neale and Kinlaw, 1991). The quality and quantity of forest trees can be improved via molecular genetic studies of gene manipulation and gene improvement of forest trees. According to FAO World Bank Development report, demand for wood product is increased from 3.5 billion m^3 in 1990 to 6.4 billion m^3 in 2020.

The Sarawak State Government is aimed to develop one million hectares of planted forests tree in year 2020 through cooperation between Sarawak Timber Association (STA) and Sarawak Forestry (Datu, 2011), and *Neolamarckia cadamba* is selected as one of the tree species by the Sarawak State Government. However, little is known about this species despite its usage and economic value. To date, Ho *et al.* (2012) had been conducting extensive molecular works on this species, in which major interest are on the (1) genetic diversity of *N. cadamba* in natural and planted forests in Sarawak; (2) development of simple sequence repeat DNA markers specific for genotyping *N. cadamba*; (3) the one step "Touch-incubate-PCR" approach for preparing plant tissues for high throughput genotyping of *N. cadamba*, and (4) transcriptomics and bioinformatics on wood formation of *N. cadamba*.

Neolamarckia cadamba or also known as “Kelampayan” or “Kadam” is a tropical forest tree belongs to the family of Rubiaceae. It is a woody and dicotyledon plant species. *N. cadamba* grow in wide range of soil, with the maximum growth height of about 45 m. *N. cadamba* is well known throughout the world for its wide range of values and usage. For example, leaves and barks are used in medicine, lightwood for producing low and medium quality paper, and some light construction work (Joker, 2000).

Pest and pathogen (fungi, bacteria, viruses) infections are the main problems faced by forest plantations. The ripe fruit of *N. cadamba* is attacked by insects and cause the black spot on the surface of fruit (Joker, 2000). According to the Chung *et al.* (2009), there are more than 15 insects species that infect the *N. cadamba* were identified and recorded. Common insect pests found in Sandakan, Sabah, are *Arthroschista hilaralis*, hawkmoth caterpillar (*Daphnis hypothous*), various species of bagworms, species of social caterpillar, and *Metanastria gemella*; whereas the *Endoclita aroura*, a kind of Lepitopteran stem borers is identified in Sarawak. *Arthroschista hilaralis* and *Daphnis hypothous* are called as defoliators (Chey, 2001), as it only affect growth and health of *N. cadamba* without killing the plants. Stem borer, mostly are moths in larval stage that bores in plant stems and cause the serious wood production lost of *N. cadamba*. *Endoclita aroura* and *Xyleutes ceramic* are the examples of stem borers, whereas *Hypsipyla robusta* is a kind of mahogany shoot borer (Chey, 1996). According to Chey (1996), *Hybogaster* sp. is a parasitoid on *Endoclita aroura*.

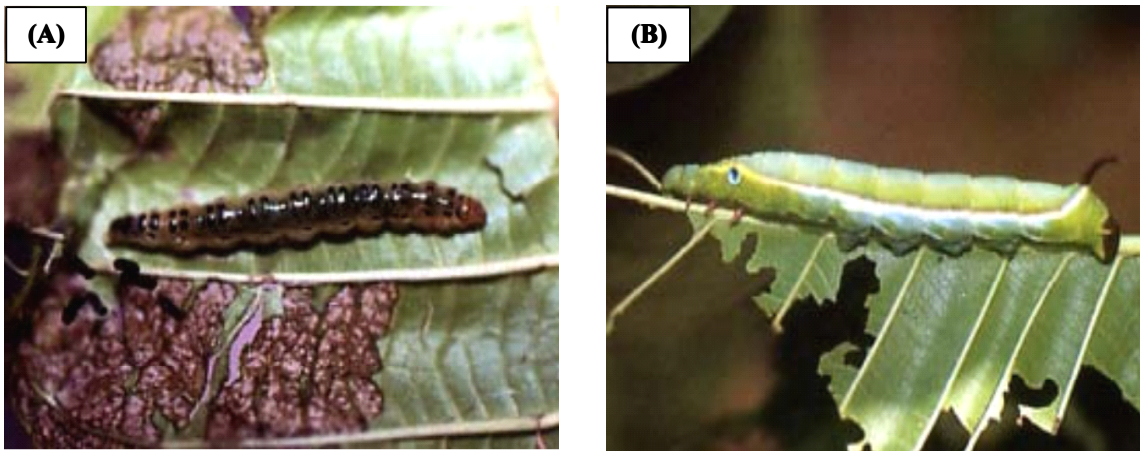

Figure 1.0: Defoliators (A) *Arthroschista hilaralis* (B) *Daphnis hypothous* (Adapted from Chey, 2001).

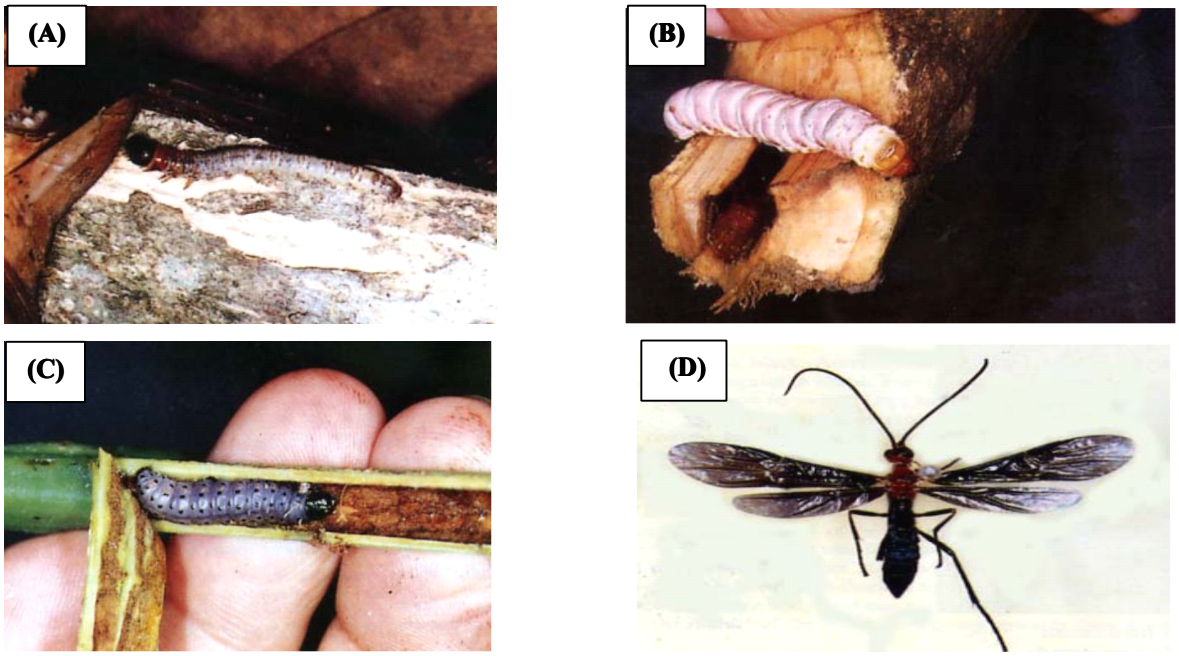

Figure 1.1: Stem-/Shoot-Borers (A) *Endoclita aroura* on teak (B) *Xyleutes ceramica* on teak (C) *Hypsipyla robusta*, mahogany shoot-borer (D) *Hybogaster* sp., parasitoid on *Endoclita aroura* (Adapted from Chey, 1996).

In the early study of transgenic plant, the gene of *Bacillus thuringiensis* (Bt) from bacterium was first introduced and expressed in tobacco plant (Carlini and Grossi-de-Sá, 2002). However, insects have evolved and developed resistancy against Bt. In addition, this technology is not accepted by the consumer due to the safety concern. Thus, alternative approach was introduced to solve the pest infection problem.

Plants have developed pest resistance during evolution in order for it to survive. The development of natural protective mechanisms such as plant proteinase inhibitors (PIs), alpha-amylase inhibitor, and hydrolyzing enzymes are the important tools used to defend against pest (Mehrabadi *et al.*, 2012). Various studies were carried out to determine the self-protective ability of plants by natural protective mechanism. For example, *Amaranthus hypocondriacus* known for having alpha-amylase inhibitor in it seeds, which specifically inhibits alpha-amylase of insects (Chagolla-Lopez *et al.*, 1994). Besides that, research has been done in plant proteinase inhibitors (PIs) and reported that PIs often present in seeds and induced in certain tissues under various stress-prone conditions such as herbivory or wounding (Macedo and Freire, 2011).

Alpha-amylase inhibitor in plants, especially in the starchy rich plant, act as bio-insecticides by inhibiting or altering the activities of alpha-amylase located in the insect's gut. Alpha-amylase is the hydrolyzing enzymes which convert the ingested starch, storage carbohydrate of plants, to energy by breaking down the starch into simple units. When the activity of alpha-amylase is inhibited, insects will fail to obtain sufficient metabolic energy. Alpha-amylase inhibitor can apply in medical and agriculture field. For example, alpha-amylase inhibitor is used to treat diseases such as obesity and hyperglycemia.

Neolamarckia cadamba is selected in this study due to its fast grow rate and economic importance. In this study, two different types of samples were use, which are developing xylem tissues and leaves. RNA was first extracted from both the developing xylem tissues and leaves of *N. cadamba* and was reverse transcribed into cDNA by using Ready-To-Go You Prime First-Strand Beads. Gradient PCR was carried out to obtain the optimum parameters. The subsequent PCR optimization was carried out in order to amplify the desired PCR product. The desired PCR product was then purified and sent for automated sequencing.

To date, there is no research done to identify the presence of alpha-amylase inhibitor gene in *N. cadamba.* Concerning the health issue of forest plantations, a lot effort has been put in order for parallel discovery of defense mechanisms in plants. Therefore, the objectives of this research are to isolate and obtain the partial cDNA of the natural alpha-amylase inhibitor gene from *Neolamarckia cadamba,* to perform *in-silico* analysis of alpha-amylase inhibitor gene based on the partial cDNA sequence generated in the present study of different plant species.

2.0 LITERATURE REVIEW

2.1 Selection of species studied (*Neolamarckia cadamba*)

2.1.1 Background of *Neolamarckia cadamba*

Neolamarckia cadamba (Roxb.) Bosser is the forest tree belongs to the family Rubiaceae. The synonyms of *N. cadamba* are *Anthocephalus chinensis* auct, *Anthocephalus cadamba* (Roxb.) Miq., *A. indicus* A. Rich., *A. morindaefolius* Korth (Joker, 2000). It has several common names depend to the place, such as "Kadam" named by Indian and French, "Kaatoan bangkal" in Philippines, "Kelampayan" in Malaysia and "thkoow" in Cambodia. *N. cadamba* is widely distributed and grow naturally from India, Nepal and India, through Thailand and Indo-China and eastward in the Malaysian Archipelago to Papua New Guinea (Joker, 2000). Plantations have been raised in India, Sri Lanka, Myanmar, Indonesia, Malaysia and the Philippines (Nair, 2007). According to Nair (2007), *N. cadamba* is planted in Java, Indonesia to replace poor teak plantations after harvest, and in North Sumatra, Riau and Central Kalimantan, it is an industrial plantations species for pulpwood production. In addition, *N. cadamba* also planted in tropical and subtropical countries, such as in South Africa, Puerto Rico, Surinam and Taiwan (Nair, 2007).

2.1.2 Characteristics description of *Neolamarckia cadamba*

N. cadamba is a woody and dicotyledon forest trees. It is common in logged-over lowland dipterocarp forests and growth well in freshwater swamps (Nair, 2007). It can attain 45 m of height, without branches for more than 25 m with diameter up to 160 cm (Lim *et al.*, 2005; Peter, 2007). Its branches are arranged in tiers with 13 to 32 cm long of leaves. Its flower has diameter of 3 mm, yellow-orange in colour; small, globose heads which arrange

in dense with corolla tube. Joker (2000) found that *N. cadamba* start flowering when grown up in 4 to 5 years old. Fruit body has about 45 mm diameter, ripe fruit will change colour to dark brown and the seeds inside fruits are matured. Seeds are transferred to other places by wind or rain dispersion, floods and rivers. The habitat of *N. cadamba* has found below 1000 m altitude (Lim *et al.*, 2005) in secondary forest. It favors in the habitat where there is more than 1500 mm rain per year. However, some are found growing in the dry place with minimum 200 mm rain per year (Joker, 2000). *N. cadamba* manages to grow in a wide variety of soils and able to tolerates with the periodic flooding. Figure 2.0 shows the different parts of *N. cadamba*.

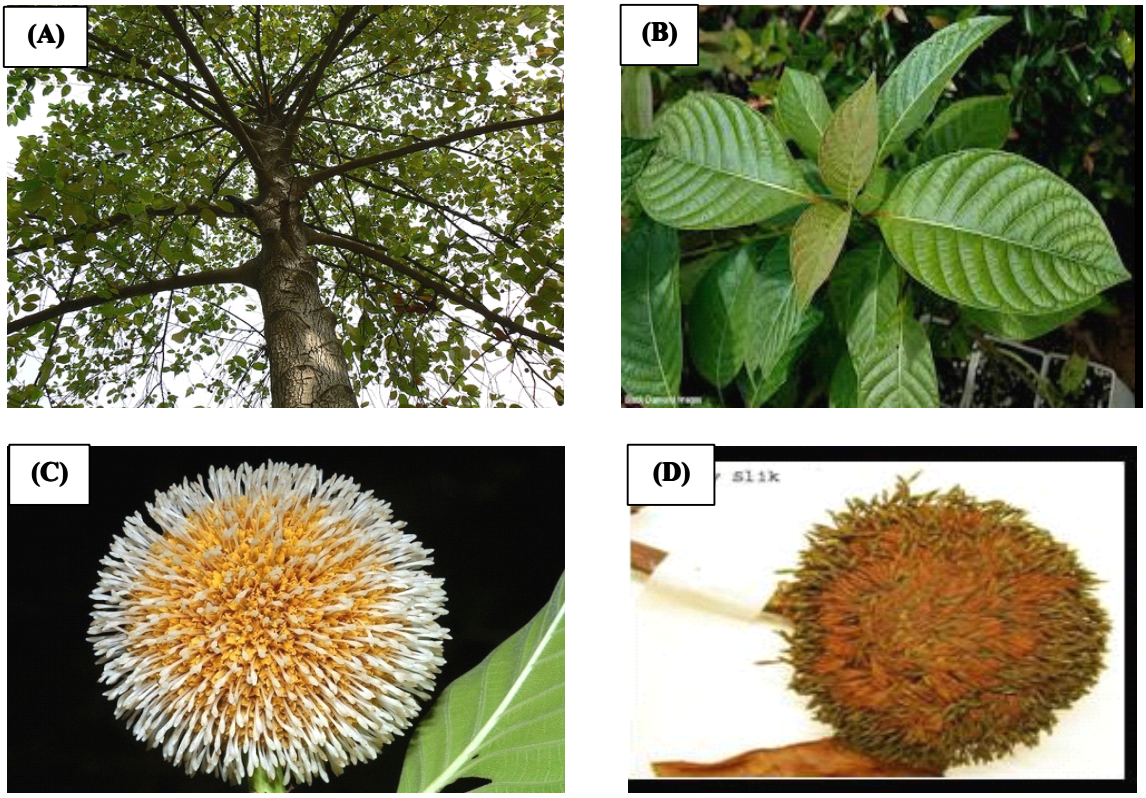

Figure 2.0: Different parts of *Neolamarckia cadamba.* (A) Branches of *N. cadamba* arrange in tiers (B) Leaves of *N. cadamba* (C) Flower of *N. cadamba* arrange in dense (D) Dark brown color of ripe fruit (Pictures adapted from https://www.google.com.my/search?q=Neolamarckia+cadamba&hl=en&biw=1280&bih=699&prmd=imvns&tbm=isch&tbo=u&source=univ&sa=X&ei=9cpVUKH8J4yurAecl4DgCg&sqi=2&ved=0CC4Qs).

2.1.3 Uses and importance of *Neolamarckia cadamba*

Fast growing rate and the significant values of *N. cadamba* has attracted many researchers to give much concern to this tree, especially local government, such as Sabah Forestry Department and Sarawak Government (Chung *et al*., 2009). *N. cadamba* is well known for its high economically values due to its wide uses, such as timber use for plywood manufacture, packing cases, wooden sandals, toys and disposable chopsticks (Choo *et al*., 1999), picture frames, low and medium quality paper from pulp and light construction work (Joker, 2000). It also possesses medicinal properties where the bark of the trees can be used to release fever, production of astringent anti-hepatotoxic and anthelmintic (Kapil *et al*., 1995). Bussa and Pinnapareddy (2010) reported that *N. cadamba* is used to treat for

various diseases, including diabetes mellitus, venereal disease and peptic ulcers. Due to its economical and medical values, *N. cadamba* has become the priority species for large scale tree plantations in Sarawak (The star online, 2011).

2.2 Alpha-amylase

Alpha-amylase, also known as 1, 4-α-D-glucanohydrolase (EC 3.2.1.1), is common monomeric enzyme (Kadziola *et al.*, 1998) found in both higher and lower plants, animals, microorganisms and insects (Carlini and Grossi-de-Sá, 2002). Alpha-amylase is type of digestive enzymes which role in breaking-down the complex carbohydrates into simple sugars. Its main function is to facilitate the inter-conversion of various oligosaccharides or polysaccharides into maltose units by catalyzing the α-1, 4-glucosidic linkages (Prince, 1999). Alpha-amylase is important enzyme for most organisms to obtain the energy through carbohydrate metabolism (Franco *et al.*, 2002). Alpha-amylase is found in salivary gland and the pancreas in animals; whereas in plants, alpha-amylase is produce during germination of seeds (Hill and MacGregor, 1988). It is a pH and temperature dependent enzyme, which requires optimum condition to carry out its activity (Amutha and Jaya Priya, 2011). Activities of alpha-amylase are applied in industry and medical field. For examples, in baking industry, this enzyme is used to improve taste, colour and toasting quality of bread; whereas in medical field, alpha-amylase activity is inhibited in order to treat diseases, such as hyperglycemia and diabetes (Prince, 1999). Due to the important values of alpha-amylase, the structure and mechanisms of alpha-amylase have been studied.

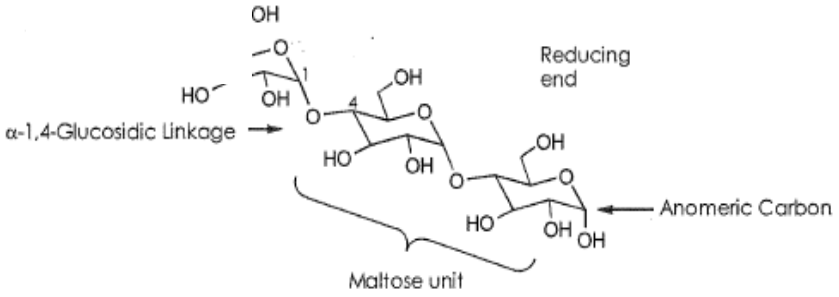

Figure 2.1: Diagram of alpha-amylase breakdown the glucosidic linkages between C1 and C4 of two adjacent glucose molecules
(Adapted from Prince, 1999).

2.2.1 Alpha-amylase in insect

Insect alpha-amylase has molecular weight ranged from 48 to 60 kDa, isoelectric point (pI) of 3.5-4.0, Km values around 0.1% and the pH values depend with the location of amylase isolated (Krystal Worthington, 2012). The optimum pH of insect alpha-amylase is varied from acidic to alkali. According to Sivakumar *et al.* (2006), *Callosobruchus chinensis, Tribolium castaneum* and *Sitophilus oryzae* are the examples of insects which have acidic pH optima of alpha-amylase. Sivakumar *et al.* (2006) also reported that alpha-amylase of phytophagous Lepidopteran insects show high enzyme activity in alkali pH, similar to *Plodia interpunctella, Ephestia kuehniella* and *Corcyra cephalonica* larvae. Activity of alpha-amylase in insect also depends to temperature. Alpha-amylase from Coleopteran insects and Lepidopteran insects show high enzyme activity at the temperature range from 30-40 ^{o}C; whereas *T. castaneum*, show high alpha-amylase activity at 60 ^{o}C.

Insects are polysaccharide-rich diet organisms which depend on their own alpha-amylase, located in the middle of gut of insects, which use to catalyze α-D-1, 4 glucosidic linkages that found in starch components. According to Ophardt (2003), they found that

the cereal grains and tubers are rich in starch. There are two main polysaccharides which make up of starch, namely amylose and amylopectin. Amylose is an unbranched molecule in which the glucose units are connected each other by α-1, 4-glucosidic linkages. Alpha-amylase catalyzes the linkage, convert amylose into maltose. On the other hand, amylopectin is a highly-branched molecule in which the chain is connected each other by α-1, 6-glucosidic linkages. Alpha-amylase catalyzes amylopectin into maltose and small α-dextrin. Mehrabadi *et al.* (2012) reported that seed weevils are the examples of starch dependent insects which feed starchy seeds during larval or adult stages. Alpha-amylase is the important hydrolytic enzyme for seed weevils to obtain energy source through carbohydrate metabolism.

2.2.2 Structure of insect alpha-amylase

The three dimensional structure of *Tenebrio molitor* enzyme (TMA) has been determined (Franco *et al.*, 2002). This enzyme show high activity in acidic environment with pH optimum of 5.8 in order for cleavage of the glycosidic bond to occur. It is made up of one polypeptide chain with 471 amino acid residues, one calcium ion, one chloride ion and 261 water molecules (Franco *et al.*, 2002). Insect alpha-amylase is calcium dependent and is activated by chloride (Mehrabadi *et al.*, 2012). Both calcium and chloride ions are located close to the active site of enzyme (Prince, 1999). Calcium ions play an important role to maintain the integrity of enzyme by regulates the correct positions of catalytic residues (Prince, 1999). Glutamic acid (Glu) and aspartic acid (Asp) are the catalytic residues which located in the active site of enzyme (Payan, 2003).

Three distinct domains, namely domain A, domain B and domain C, have been observed due to the protein fold in TMA (Mehrabadi *et al.*, 2012). Prince (1999) reported